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Jean C Lee

Attorney Docket No.: 13353.1073
PATENT APPLICATION

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of **Matthew Glenn, Ilkka Havukkala, Mark William Lubbers, and James Dekker**

Group Art Unit: 1652

| | | |
|---|---|---|
| Application No. | : | 10/650,274 |
| Filed | : | August 28, 2003 |
| For | : | **POLYNUCLEOTIDES AND POLYPEPTIDES, MATERIALS INCORPORATING THEM AND METHODS FOR USING THEM** |
| Examiner | : | Malgorzata Walicka |

## DECLARATION OF DR. JAMES DEKKER

MS: AMENDMENT
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

The undersigned, Dr. James Dekker, hereby declares:

1. I am presently a Senior Research Scientist at Fonterra Co-Operative Group, the assignee of the subject patent application, and an inventor of the claimed subject matter. I have a PhD in the field of molecular biology and immunology. The following studies were carried out under my supervision.

2. Pyruvate oxidase (EC 1.2.3.3) acts as an oxoreductase with activity towards pyruvate and other α-keto acids. It requires thiamine diphosphate and flavin adenine dinucleotide (FAD) as co-factors, and also exhibits decarboxylase activity as it catalyses the release of carbon dioxide ($CO_2$) from the α-keto acid pyruvate.

Comparison of predicted amino sequences of the pyruvate oxidase enzyme from *Lactobacillus plantarum* (POW) and AP4 of *L. rhamnosus* HN001 (SEQ ID NO: 172 of the instant specification) showed that most of the amino acids important for co-factor binding and enzyme activity in *L. plantarum* pyruvate oxidase were present in the AP4 amino acid sequence, indicating that the AP4 nucleotide sequence of SEQ ID NO: 73 encoded a pyruvate oxidase (Exhibit 1).

More specifically, Exhibit 1 shows an alignment of predicted amino acid sequences of pyruvate oxidase genes from *Lactobacillus plantarum* (GenBank accession no. 1POW_B; 1POW) and *Lactobacillus rhamnosus* HN001 SEQ ID NO: 172 (HN001_AP4). Identical amino acids at each position are shaded. Most of the amino acids found to be important for 1POW substrate binding and enzyme activity according to X-Ray crystallographic studies (Muller YA, Schumacher G, Rudolph R, Schulz GE; *Jnl. Mol. Biol. 237*:315-335, 1994) are preserved in AP4. Symbols under the aligned amino acid sequences indicate 1POW amino acids found to be important for glycerol binding (Δ), thiamine diphosphate binding (•), FAD binding (▲), enzyme active site (♦), and metal ion binding (◊). Dotted line above the alignment indicates the presence in both sequences of Prosite motif PS00187, a signature of thiamine pyrophosphate enzymes ([LIVMF]-[GSA]-x(5)-P-x(4)-[LIVMFYW]-x-[LIVMF]-x-G-D-[GSA]-[GSAC]).

To determine whether the enzyme encoded by the AP4 nucleotide sequence exhibited decarboxylase activity toward α-keto acid substrates, the full-length polynucleotide sequence of pyruvate oxidase AP4 from *L. rhamnosus* strain HN001, given in SEQ ID NO: 73 was amplified from *L. rhamnosus* HN001 DNA using standard PCR methodology. AP4 PCR product was then cloned into pFRC019, an in-house expression vector, and transformed into *L. rhamnosus* HN001.

The α-keto acid decarboxylating activity of AP4 was tested using three substrates, α-ketoisocaproate, α-ketomethylvalerate, and α-ketoisovalerate that yield 3-methylbutanal (3MB), 2-methylbutanal (2MB), and 2-methylpropanal (2MP), respectively, following α-keto acid decarboxylation. Cell-free extracts were prepared from *L. rhamnosus* HN001 carrying the AP4 genetic construct, *L. rhamnosus* HN001 carrying empty pFRC019 expression plasmid vector, *L. rhamnosus* HN001 carrying a genetic construct for an irrelevant protein, and a *Lactococcus lactis* strain B1113

known to exert strong α-keto acid decarboxylating activity as a positive control. In all cases, cell extracts were prepared from late log-phase cells grown in M17 media using a French pressure cell at 18000 psi, and the supernatants filtered through a 0.45 μm hydrophilic membranes (Millipore). A time-course assay of α-keto acid decarboxylating activity was performed at 30 °C, pH 6.3, using 20 mM of the different α-keto acid substrates and other reaction conditions as described by de la Plaza et al. (*FEMS Microbiol. Lett.* 238:367-74, 2004). As an internal standard, *Lactococcus lactis* MG5295 extracts were spiked with 20–100 μM of the expected reaction products. Enzyme activity was assayed over a 6 h period by fast GCMS headspace analysis in 1.5 ml vials using set ion m/z ratios of 58 for 3-methylbutanal, 57 for 2-methylbutanal and 72 for 2-methylpropanal. Peak height data from chromatograms were plotted vs. time and used to calculate enzyme activities as nmol/min/mg total protein (Table 1).

**Table 1.** Decarboxylase enzyme activity on three α-keto acid substrates. Numbers show enzyme activity as production of each reaction product (nmol/min/mg total protein) assayed over 6 h.

| Cell extract | 3-Methylbutanal | 2-Methylbutanal | 2-Methylpropanal |
|---|---|---|---|
| *L. rhamnosus* HN001 + vector only | 0.002 | 0.002 | 0.002 |
| *L. rhamnosus* HN001 + irrelevant protein | 0.001 | 0.002 | 0.003 |
| *L. rhamnosus* HN001 + AP4 | **0.028** | **0.048** | **0.044** |
| *L. lactis* B1113 | 0.188 | 0.137 | 0.496 |

As shown in Table 1, while cell extracts of *L. rhamnosus* HN001 carrying empty expression vector or expression vector encoding an irrelevant protein showed no significant decarboxylation of the α-keto acid substrates, the cell extract of *L. rhamnosus* HN001 containing the expression vector encoding pyruvate oxidase AP4 showed significant enzyme activity toward all three substrates. Therefore, based on both amino acid comparison and enzyme activity assays, the *L. rhamnosus* HN001 gene *AP4* (SEQ ID NO: 73) encodes pyruvate oxidase.

3. The undersigned further declares that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful, false statements, and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 35 of the United States Code.

James Dekker, Ph.D.

2/7/06

Date

**Exhibit 1**

```
          1                                                  50
    1POW  MVMKQTKQTN ILAGAAVIKV LEAWGVDHLY GIPGGSINSI MDALSAERDR
HN001_AP4 .MEASVMTEK INAADAMIKV LEDWGIHNIY GLPGGSFDST MNALYNRRHT
                                          ΔΔ

         51                                                 100
    1POW  IHYIQVRHEE VGAMAAAADA KLTGKIGVCF GSAGPGGTHL MNGLYDARED
HN001_AP4 INYVQVRHEE VGALAAAGEA KVTGRIGATF GSAGPGAVHL LNGLYDAQYD
               **                                 *       *  ▲▲

         101                                                150
    1POW  HVPVLALIGQ FGTTGMNMDT FQEMNENPIY ADVADYNVTA VNAATLPHVI
HN001_AP4 HVPVLALVGQ VPTAAMNTNY FQEMNENPMF ADVSVYNRTA MTAAQLPHVV
          ▲                   ♦*

         151                                                200
    1POW  DEAIRRAYAH QGVAVVQIPV DLPWQQIPAE DWYASANSYQ TPLLPEPDVQ
HN001_AP4 DEAIRQAYKY QGVAVVTIPK DLGWQEI.DD NYVSSANLYQ KPLLPEPDPE


         201                                                250
    1POW  AVTRLTQTLL AAERPLIYYG IGARKAGKEL EQLSKTLKIP LMSTYPAKGI
HN001_AP4 QVATAWSILK DAKKPILYVG NGARGARDEI IAFSEKTHIP IITTALAKGV
                        ▲   ▲                      ▲▲▲

         251                                                300
    1POW  VADRYPAYLG SANRVAQKPA NEALAQADVV LFVGNNYPFA EVSKAFKNTR
HN001_AP4 VPDDYKANMG SAGRVASKPG VEVARGADTV LFLGSDFPFQ PYFIAPN..A
                  ▲ ♦▲                    ▲ ▲  ♦
                    ▲

         301                                                350
    1POW  YFLQIDIDPA KLGKRHKTDI AVLADAQKTL AAILAQVSER ESTPWWQANL
HN001_AP4 KYIQVDIDAS KFGRRHTVDL AVLADAKKFI KALTEKADAV PETAWYRAAV
            ▲▲     ▲             ▲▲

         351                                                400
    1POW  ANVKNWRAYL ASLEDKQEGF LQAYQVLRAV NKIAEPDAIY SIDVGDINLN
HN001_AP4 ANKANWAEWM TSFEDDSQTP LRVEPIFKLI NEMADKDAVF QVDVGNVTIN
                                                    * **

         401                                       ----------450
    1POW  ANRHLKLTPS NRHITSNLFA TMGVGIPGAI AAKLNYPERQ VFNLAGDGGA
HN001_AP4 GMRYLKANDN QIFTTSGWYA TMGYAVPAAI GAQAEFPDRQ VWSISGDGGF
                        ▲  *  *                          ◊**
                                                         *

         451                                                500
    1POW  SMTMQDLATQ VQYHLPVINV VFTNCQYGFI KDEQEDTNQN DFIGVEFNDI
HN001_AP4 AMVMQDIMTQ VKYHMPIINI VLTNESLGFI EAEQDDTRQP .HSGVDLIDA
          ◊  ◊                  * ◊ * ♦**   Δ
                                  *   *

         501                                                550
    1POW  DFSKIADGVH MQAFRVNKIE QLPDVFEQAK AIAQHEPVLI DAVITGDRPL
HN001_AP4 DYGKAAEAMG AQGFEVHNLD ELKAAFAKAK ..DRKGPVVI DVKISDLRPI


         551                                            597
    1POW  PAEKLRLDSA MSSAADIEAF KQRYEAQDLQ PLSTYLKQFG LDD....
HN001_AP4 PVEQLVLDKQ TQDPEAVDAF VKKYHAETLI PFGSYSRMLK RQLQTFN
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